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Evaluation of the relationships between biochemical endpoints of PAH exposure and physiological endpoints of reproduction in male California Halibut (*Paralichthys californicus*) exposed to sediments from a natural oil seep

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Abstract

Coal Oil Point (COP) is a natural oil seep off the coast of Santa Barbara, California. Although most studies examining the fate and effects of petroleum have focused upon urbanized or anthropogenic sources of inputs, few have examined the effects of polyaromatic hydrocarbons (PAHs) derived from natural seeps. In order to evaluate the effects of PAHs derived from COP on marine fish populations, hatchery-reared California Halibut (*Platichthys californicus*) were exposed for 30 days to seven dilutions of sediments collected from COP. Hepatic cytochrome P450 1A (CYP1A), biliary fluorescent aromatic compounds (FACs), gonadal somatic indices, and plasma steroid concentrations. Sixteen USEPA priority PAHs were targeted for analysis in each sediment dilution. In general, biochemical responses were somewhat recalcitrant to dose–response relationships and were less sensitive than the literature values established for the same indicators following exposure to urbanized PAHs. Trends toward reductions in plasma 17β-estradiol concentrations were observed, but reductions in gonadal somatic indices were not observed. FAC values for naphthalene, benzo(a)pyrene,

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phenanthrene-related compounds reached maximums at 33–100% COP sediment. The resulting insensitivity may be unique for exposure to "natural" petroleum due to a higher concentration of lower molecular weight PAHs or uncharacterized inhibitors.

Keywords: PAHs; Oil seep; CYP1A; FACs; GSI; Sex steroids

1. Introduction

Input of polyaromatic hydrocarbons (PAHs) into the marine environment continues to increase (Brown et al., 1998; Law, 2000). Although anthropogenic sources are thought to dominate environmental input, natural oil seeps can also contribute a significant amount to local environments (Blumer & Sass, 1972; Reed, Kaplan, Sandstrom, & Mankiewicz, 1977; Wijayaratne & Means, 1984). Seepage from the natural oil seep near Coal Oil Point (COP), off the coast of Goleta/Santa Barbara, California has been estimated to have been occurring for thousands of years at 95,000–19,000 l/day of petroleum (Allen, Schlueter, & Mikoloj, 1970; Simoneit & Kaplan, 1980). In contrast to urbanized PAHs, low molecular weight hydrocarbons tend to be the primary constituents of the seep material (Spies, Davis, & Stuerner, 1980).

Indicators of PAH bioavailability in several studies around the world in various fish species include a variety of biochemical, histological and physiological endpoints (Aas, Baussant, Balk, Liewenborg, & Andersen, 2000; Arinc & Sen, 1999; Kirby et al., 1999; Miller et al., 1999; Monteiro et al., 2000; Petersen & Kristensen, 1998; Teles et al., 2003). These indicators of exposure have been linked to various effects such as carcinogenicity, hepatic lesions, precocious and inhibited gonadal development, alterations in egg size and number, inhibited spawning, morphological abnormalities, and reduced egg and larval viability (Casillas et al., 1991; Collier et al., 1992; Incardona, Collier, & Scholz, 2004; Johnson, Casillas, Collier, McCain, & Varanasi, 1988, 1993, 1997, 1999). However, most of these studies evaluated urbanized multi-ring PAHs resulting from anthropogenic inputs.

Previous studies in feral fish collected from the COP area demonstrated enhanced hepatic cytochrome P4501A (CYP1A) expression and biliary fluorescent aromatic compounds (FACs), indicative of PAH bioavailability (Roy et al., 2003; Spies et al., 1996). Seven day exposure studies by Roy et al. (2003) examining biochemical responses in naïve Hornyhead Turbot (*Pleuronichthys verticalis*) exposed to COP sediments indicated enhanced CYP1A and FAC concentrations, but at sediment PAH concentrations significantly higher than sediment thresholds suggested in urbanized areas (Collier et al., 2002). In contrast, serum estradiol concentrations were significantly reduced following sediment-only exposures at the lowest PAH dilution which was $\approx$12 μg/g of 16 PAHs listed as USEPA priority pollutants (Roy et al., 2003).

To determine the potential effects of diminished steroid concentrations on higher level responses in flatfish, cultured male California Halibut (*Paralichthys californicus*)

were exposed to 7 concentrations of COP sediments for 30 days. Gonadal somatic indices, plasma steroid concentrations, hepatic CYP1A and biliary FACs were measured to determine whether relationships existed between biochemical and physiological endpoints. California Halibut are benthic flatfish which inhabit estuaries and nearshore coastal areas as larvae and juveniles, then increase in spatial distribution offshore with increasing age. California Halibut are also an economically important species, being on average the 6th most valuable vertebrate catch off the California coast. Revenue for 2002 was $2,800,000 for California Halibut, with an average increase of about 10% per year for the past three years (http://www.st.nmfs.gov/; Personal communication from the National Marine Fisheries Service, Fisheries Statistics and Economics Division, Silver Spring, MD). Exposures were conducted with the aim of determining dose response concentrations which may be used in helping to set sediment thresholds for adverse effects and help in assessing locations undergoing remediation.

2. Materials and methods

2.1. Exposure system

PAH contaminated sediment was collected from the natural marine oil seep COP in Goleta (Santa Barbara) (119.53.428 longitude; 34.24.370 latitude), California as previously described (Roy et al., 2003). Exposure sediments contained 0%, 0.33%, 0.66%, 1%, 33%, 66%, and 100% COP sediments diluted to their respective nominal concentrations with reference sediment from Orange County, California (118.05.1999 longitude; 33.36.055 latitude). Sediments were extensively homogenized in 41 glass beakers until all aggregates were no longer visible. Due to consistency restraints, total organic carbon (TOC) and grain size could not accurately be determined from COP sediments. The TOC of the reference sediments were $0.42 \pm 0.004\%$. The composition was 73.8% sand; 21.3% silt and 4.9% clay with a median grain size of 3.68 (Roy et al., 2003). Exposures were conducted at the Sea Laboratory in Redondo Beach, California using filtered ambient seawater ($18 \pm 1\,^{\circ}C$) from inflow pipes from the open ocean. Aquaria were housed in shaded outdoor areas receiving indirect sunlight in June of 2003 under natural light:dark conditions.

Sexually mature male California Halibut (56.5 ± 5.0 cm; 550 ± 52 g) were generously donated by Hubbs SeaWorld Research Institute (Carlsbad, CA). Fish were acclimated to the facilities at the Sea Laboratory for over two months in a circular tank of $\approx 12\,m^3$. Prior to the addition of fish, ≈ 21 of sediment were transferred into 40 l glass tanks under flow-through conditions and the sediment was allowed to settle. Two fish were placed in each aquarium, with two replicates per exposure. Fish were fed pellet food daily and exposures were conducted for 30 days.

Following 30 days, fish were transferred to containers having 10 l of seawater with 10 g/l of MS 222. Following anesthesia, the fish were weighed and the lengths measured. Blood samples were collected from the spinal caudal artery with a syringe, and immediately centrifuged at 750*g* to separate serum/plasma. Serum/plasma samples

were placed in separate tubes and frozen on dry ice. Length measurements were taken from the tip of the lower mouth to the end of the tailbone where the back fin begins to scale differently than the body scales. Backbones were severed with a knife, and liver and bile samples were removed, placed in cryogenic tubes, and immediately placed in dry ice for transport to a −80 freezer.

2.2. Chemical evaluation of PAHs in sediments

Chemical analyses of sediment were carried out as reported in Roy et al. (2003). Samples were stored at 4 °C until analysis. Sixteen USEPA priority PAHs were measured as described in EPA method 8100 (USEPA, 1996). Sediment samples were extracted with hexane using an ultrasonic disruptor (550 Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA). Cleanup was performed with fully activated silica gel (8 g), conditioned with hexane, and PAHs were collected from the column with 25 ml of methylene chloride/hexane (2:3, v:v) A GC-FID (flame ionization detector) with a capillary column (DB-5) was used for analysis and quantification. The oven temperature was 40 °C, ramped to 160 °C with 40 °C/min, and up to 300 °C with 5 °C/min. The recoveries were 30–111% with a SD of 2–15% and MDLs ranging from 11 to 53 ng/g sediments. Selected PAHs were only a small representative of the total PAHs present.

2.3. Biochemical endpoint measurements

2.3.1. Blood steroid analysis

17β-Estradiol (E2) and Testosterone (T) concentrations were analyzed spectrophotometrically using immunoassay kits from Cayman Chemical Company (Ann Arbor, MI) following the manufacturers instructions. Approximately 50 μl of serum/plasma was used for triplicate analyses. Testosterone measurements did not include 11-keto testosterone.

2.3.2. FAC analysis

Gall bladders were thawed on ice, and bile removed. Samples were diluted in 99% HPLC- grade methanol and fluorescence measured with a Shimadzu fluorescence detector (RF-10 AXL) at 380/430, 256/380, and 290/335 nm excitation/emission for benzo(a)pyrene (BAP), phenanthrene (PHN) and naphthalene (NAP) like compounds, respectively. Concentrations were calculated using standard curves developed from PHN, NAP, and BAP standards.

2.3.3. CYP1A analysis

Liver microsomal fractions were obtained by ultracentrifugation as previously described (Roy et al., 2003). Total protein concentrations for these fractions were attained by utilizing the Pierce kit BSA standards. CYP1A protein was measured using 50 μg of protein per sample and conducting western blots. Western blot detection was carried out using a primary anti-fish CYP1A monoclonal antibody, C10-7 from Biosense Laboratories (Bergen Norway). Proteins were initially separated by

SDS–PAGE, then transferred overnight to nitrocellulose. The nitrocellulose was incubated with blocking buffer (0.5 g nonfat dried milk in 50 ml phosphate buffer solution) in a heat-sealed bag for 1 h, then incubated with primary antibody followed by secondary anti-mouse alkaline phosphatase, each for 1 h. The primary and secondary antibodies were each diluted 1000× in blocking buffer. Four fifteen minute rinses with PBS were completed in between each incubation. Following colorimetric detection, band optical densities were quantified using QuantityOne software from BIORAD (Hercules, CA). Proteins were measured using the Pierce reagent (Pierce Inc., Rockford, IL).

2.3.4. Statistical analysis

Bartletts's test of homogeneity was conducted to verify homogeneity of variance. If data were homogeneous, and treatment groups possessed an N of at least 3, one-way analysis of variance (ANOVA) was conducted to determine possible differences between groups. A Bonferonni post hoc test was utilized with $p \leqslant 0.05$ employing GraphPad Prism 3 software (San Diego, CA).

3. Results

Total concentrations of selected PAHs ranged from 0.171 to 107.6 μg/g (Table 1). In the 0.66–66% COP sediments, acenapthene was the dominant PAH with an average concentration of 30 μg/g in the 100% COP treatment. Other PAHs with high relative concentrations included acenaphthylene as well as fluorene. Other notable concentrations included fluoranthene with an average concentration of 1.4 μg/g, followed by dibenzo(ah)anthracene at 7.2 μg/g, and phenanthrene at 4.4 μg/g in the 100% COP sediment.

Hepatic cytochrome P450 1A (CYP1A) expression was similar to the control values for the 33% COP sediments, with significant induction at the 66% and 100% COP sediments (Fig. 1). *P*-values were 0.05% for the 66% sediment, and 0.01 for the 100% sediment exposure.

FAC accumulation in bile was variable in the 33%, 66% and 100% PAH sediment exposures, with a trend towards increasing FACs in the bile at the higher sediment concentrations (Fig. 2). Values for BAP ranged from 5 to 30 μg/ml, PHE from 33 to 2300 μg/ml, and NAP from 500 to 14,000 μg/ml. The lower concentrations of contaminated sediment (0.33%, 0.66%, and 1%) showed a slight trend for increasing FACs with measured PAHs in the sediment exposures. Regression analyses of FACs with measured sediment PAHs in each class or replicate/treatment failed to show significant correlations ($p > 0.05$). FACs fluorescing at the PHN and NAP wavelengths were highly correlated, with an r^2 of 0.97. BAP values were less related to PHN and NAP, with r^2 values of 0.72 and 0.71, respectively.

No significant differences in plasma steroid concentrations between treatment groups and controls were observed (Fig. 3). However, a trend toward reduction of estradiol levels was observed at the 1% COP treatment. E2 concentrations were

Table 1
Sediment concentrations of selected PAHs and hydrocarbons (μg/g) in COP sediments exposed to male California Halibut for 30 days

	Percentage of COP sediment													
	Control 1	Control 2	0.30%	0.30%	0.67%	0.67%	1%	1%	33%	33%	67%	67%	100%	100%
Idene	<0.053	<0.053	0.15	0.183	0.285	0.067	0.088	0.127	0.76	0.65	0.28	0.2	0.89	0.81
Naphthalene	<0.027	<0.027	0.056	<0.027	0.065	0.069	<0.027	<0.027	1.25	0.8	0.19	0.2	4.46	4.4
Acenaphthylene	0.04	0.089	<0.038	<0.038	<0.038	<0.038	<0.038	0.061	4.42	6.6	7.93	9.16	26.18	2.47
Acenaphthene	0.04	0.092	0.019	0.059	1.143	0.387	1.284	1.047	11.8	17.88	6.3	8.76	36.61	23.15
Perylene	<0.028	<0.028	<0.028	<0.028	0.115	0.035	0.152	0.031	0.05	ND	0.05	0.02	0.15	0.33
Indeno(123cd) pyrene	<0.030	<0.030	<0.03	<0.03	<0.030	<0.030	<0.03	<0.03	2.23	1.89	2.28	5.24	0.76	3.15
Fluorene	0.03	<0.029	0.145	0.17	0.164	<0.029	0.111	0.036	2.9	3.2	4.05	7.2	9.08	12.4
Phenanthrene	<0.042	<0.042	0.084	0.11	0.059	0.044	0.099	<0.042	1.48	2.09	1.69	1.06	4.64	4.23
Anthracene	<0.048	<0.048	0.08	<0.048	<0.048	<0.048	0.093	<0.048	0.34	0.59	3.1	1.4	1.74	2.14
Fluoranthene	<0.038	<0.038	<0.038	<0.038	<0.038	0.056	<0.038	<0.038	0.77	1.13	0.8	1.5	2.01	0.78
Pyrene	0.061	<0.048	<0.048	0.112	<0.048	<0.048	0.086	<0.048	0.25	0.27	0.54	0.4	1.7	1.94
Benzo(a)anthracene	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03	0.073	<0.03	0.56	0.36	0.33	0.3	1.54	1.16
Chrysene	<0.032	<0.032	<0.032	<0.032	0.093	<0.032	<0.032	<0.032	0.15		0.21	0.14	3.98	4.35
Benzo(ghi)perylene	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	<0.025	0.52	0.24	3	2.8	0.9	0.8
Benzo(b)fluoranthene	<0.028	<0.028	0.043	<0.028	<0.028	0.074	<0.028	<0.028	0.39	0.32	0.26	0.3	0.5	0.9
Benzo(k)fluoranthene	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022	<0.022
Benzo(e)pyrene	<0.043	<0.043	0.192	0.227	0.046	0.084	0.131	0.068	0.34	0.36	0.21	0.73	3.72	3.35
Benzo(a)pyrene	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	<0.021	0.78	0.58	0.79	2.2	0.28	0.45
Dibenz(ah)anthracene	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.35	<0.02	1.88	4.76	2.3	1.4	8.35	5.98
Benzo(j)fluoranthene	<0.011	<0.011	<0.011	0.24	<0.011	<0.011	<0.011	<0.011	0.09	0.07	0.11	0.22	0.14	0.06
Total	0.17	0.18	0.77	1.10	1.97	0.82	2.46	1.37	31.0	41.8	34.4	43.2	107.6	72.9
Total average	0.18		0.85		1.39		1.92		36.4		38.8		90.2	

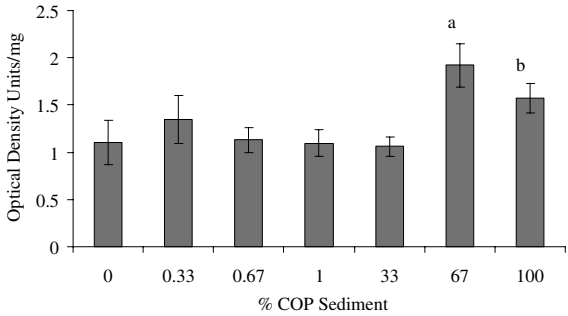

Fig. 1. Expression of hepatic CYP1A in California Halibut following 30-day exposure to various dilutions of COP sediment. Each value represents the mean ± SD of 3–4 animals. $a = p \leqslant 0.05$; $b = p \leqslant 0.01$.

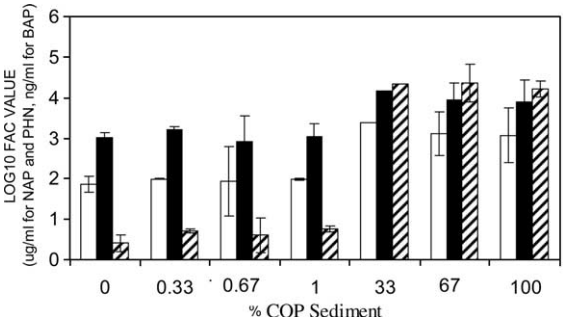

Fig. 2. Biliary FACs in California Halibut following 30-day exposure to various dilutions of COP sediment. Each value represents the mean ± SD of 1–4 animals. Values without error bars are a single individual. Clear bar = phenanthrene-like compounds; dark bar = naphthalene-like compounds; hatched bar = benzo(a)pyrene like compounds.

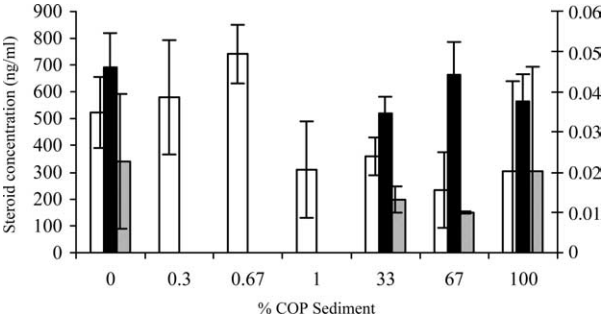

Fig. 3. Plasma steroid and gonadal somatic indices (GSI) in California Halibut following 30-day exposure to various dilutions of COP sediment. Each value represents the mean ± SD of 3–4 animals. Clear bar = estradiol concentrations (ng/ml); dark bar = GSI; hatched bar = testosterone concentrations (ng/ml).

between 501 and 720 pg/ml from the control to the 0.66% COP sediments, and from the 1 to the 100% COP sediments the values ranged from 225 to 360 pg/ml. T levels were not significantly different than controls. GSI values did not show any significant response to treatment.

4. Discussion

Benthic flatfish are included often in sediment contamination studies due to their continual exposure to sediments during their life histories. Threshold levels of contaminant exposure are needed in marine organisms to conduct accurate environmental risk assessments. California Halibut is an important marine fisheries resource economically, and they are also exposed to natural PAHs through seeps in the ocean floor along the California Coast as well as in their nurseries (estuaries). This study investigated the effects of natural PAH exposure to halibut by conducting lab exposures to several concentrations of natural PAH contaminated sediments from the oil seep COP in Goleta/Santa Barbara, California.

Attempts were made to measure 16 priority PAHs in the COP sediments and in treatment dilutions of COP sediments. The concentrations of PAHs of the 3 lowest dilutions (33%, 67%, and 100% COP) were similar to those found by Roy et al. (2003) who only evaluated 3 dilutions. The sediments for that study were collected more than a year prior to the sediments utilized for this study from the same location. Thus, there appears to be relatively little temporal variability in the concentrations of the 16 PAHs measured in sediments of the COP seep.

Although fish were exposed to sediments possessing 7 distinct PAH concentrations, no biochemical or physiological metric demonstrated a classic dose–response relationship. The LOEC of the 16 compounds for enhanced FAC responses was ≈36.3 μg/g and the NOEC was 1.9 μg/g. Since only targeted PAHs were measured, it is likely the NOEC and LOEC values of the total PAHs in the sediments are much greater than our estimates, and significantly higher than the 1 μg/g thresholds determined by Collier et al. (2002). Although FACs accumulated in the bile of Halibut following exposure to the PAH contaminated sediments, the response was not associated with the measured PAH concentrations with increases only observed at the 33, 67, and 100 COP treatments, which were not significantly different. An 18-fold increase in the sediment PAH profiles was noted between the 1, 33/67% treatments which corresponded to a 7–200-fold increase in various FACs. Measured PAH concentrations were ≈2.75-fold higher in the 100% COP sediments and FAC concentrations remained unchanged compared to the 67% animals. A similar effect was noted in previous studies with Hornyhead turbot exposed to 33%, 67%, and 100% COP sediments with a maximum response occurring at 67% without an increase at 100% COP (Roy et al., 2003). FACs have been used as indicators of PAH exposure in other species of demersal flatfish (Ariese et al., 1993; Krahn et al., 1986). FACs in fish bile accumulate with longer durations of exposure, with saturation that may or may not be species or location specific (French et al., 1996; Roy et al., 2003). Biliary FACs and CYP1A activity have been shown to be casually related with higher level effects such as neoplasia related liver lesions (Myers et al., 1998,

2003). Since FACs are highly dependent upon biotransformation and conjugation of PAHs to biliary metabolites, any alteration of biotransformation may have significant impacts on FAC formation. Numerous compounds have been shown to be substrates, inhibitors and/or inducers Phase I and Phase II enzymes. Fluoranthene has been shown to be a relatively strong inhibitor of CYP1A catalyzed oxidation of BaP (Willett, Wassenberg, Lienesch, Reichert, & Di Giulio, 2001). Alternatively, differences may be due to multiple routes of exposure (i.e., particle ingestion, skin) within the test chambers which may have prevented a proportional increase of FACs with measured PAHs. It is worthwhile to note that most previous studies have primarily focused their efforts on evaluating anthropogenic PAHs, whereas the PAHs in this study are un-urbanized. This difference in itself may be responsible for the lower than expected CYP induction. Consequently, the occurrence of complex mixtures and uncharacterized PAHs within sediments which may also act as substrates or alter metabolic processes within fish could conceivably be responsible for the lack of correlation between sediment PAH concentrations, biliary FACs and recalcitrant CYP1A induction.

Induction of CYP1A determined by protein quantification with western blots, was significant in the 66% and the 100% COP treatments. Several studies have shown marked increases in CYP1A induction in response to PAH exposure (for review see: Stegeman & Hahn, 1994). This can typically be measured by optical density analysis of western blots (as completed in our study), or measurement of the catalytic activity of 7-ethoxyresorufin *O*-deethylase (EROD). Western blots carried out by Roy et al. (2003) in Hornyhead Turbot (*Pleuronichthys verticalis*) showed similar recalcitrance with induction only in the 100% COP sediment group compared to the lower sediment exposures, which showed no presence of the protein. A study in Norway analyzing flounder (*Platichthys flesus*) and Atlantic cod (*Gadus morhua*) from various field locations found significant differences from reference and PAH-impacted areas in EROD as well as CYP activity (Beyer et al., 1996). The insensitivity of each of these responses in two separate studies using two separate fish species suggests consistent reduction in the metabolic capabilities due to exposure to COP sediments. Since the chronic toxicity of PAHs has been suggested to be mediated through bioactivation and metabolic conversion to reactive intermediates, the lack of response in naïve fish would indicate potentially lower levels of risk. However, other adverse effects, particularly targeting the endocrine and reproductive systems, may not be mediated through bioactivation. Consequently, gonadal indices and steroid concentrations were evaluated following treatment.

The gonad-somatic indexes (ratio of gonad weight to total body weight) were not associated with priority PAH concentrations or dilution treatments. Similarly, plasma steroid levels were unaltered by treatment, although a trend toward reduced E2 concentrations may be apparent. Previous studies in Hornyhead turbot observed significant reductions in circulating E2 concentrations following 7 days of treatment to 33% COP sediments (Roy et al., 2003). Response differences may be due to species sensitivities as concentrations of sex steroids in Hornyhead turbot are significantly different from California Halibut as well as English Sole (Unpublished). For example, serum E2 concentrations were higher than testosterone concentrations in controls as well as treated animals. Whether this observation is an artifact of the method utilized to measure steroids (i.e., lack of specificity with regard to the E2 antibody) is unclear as there are no

published reports of "normal" steroid concentrations in California Halibut in different developmental stages such as post spawning males. E2 concentrations in female flounder (*P. flesus*) have been shown to decrease with exposure to phenanthrene (Monteiro et al., 2000), and similar steroid inhibition has been linked to inhibition in vivo of ovarian steroidogenesis in goldfish (Evanson & Van der Kraak, 2001).

In summary, biochemical and physiological metrics evaluated in the current study failed to demonstrate dose–response relationships preventing the estimation of accurate sediment thresholds for biomarker responses. With the possible exception of depressed E2 concentrations at 1% COP sediments, no significant adverse reproductive effects were noted. Significant induction of CYP1A and a trend toward enhanced biliary FACs was observed after 30 days of exposure to 67% and 33% COP sediments, respectively. In comparison to anthropogenic PAH values seen in other studies, halibut had diminished responses in regard to FAC accumulation in bile. CYP1A induction was also insensitive with regard to the amounts of PAHs in naturally contaminated sediment from oil seeps. This may be due to a species specific low induction response, or more likely due to unique mixtures of compounds which repress or inhibit Phase I or Phase II biotransformation pathways. Studies are currently underway to explore this possibility.

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